Botulinum C2 Toxin ADP-Ribosylates Actin and Enhances O_2^- Production and Secretion but Inhibits Migration of Activated Human Neutrophils

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Abstract

The binary botulinum C2 toxin ADP-ribosylated the actin of human neutrophils. Treatment of human neutrophils with botulinum C2 toxin for 45 min increased FMLP-stimulated superoxide anion (O_2^-) production 1.5–5-fold, whereas only a minor fraction of the cellular actin pool (~ 20%) was ADP-ribosylated. Effects of botulinum C2 toxin depended on toxin concentrations, presence of both components of the toxin, and incubation time. Cytochalasin B similarly enhanced O_2^- production. The effects of botulinum C2 toxin and cytochalasin B were additive at submaximally, but not maximally effective concentrations and incubation time of either toxin. Botulinum C2 toxin also enhanced stimulation of O_2^- production by Con A and platelet-activating factor, but not by phorbol 12-myristate 13-acetate (PMA). Botulinum C2 toxin increased FMLP-induced release of *N*-acetyl-glucosaminidase by 100–250%; release of vitamin B12-binding protein induced by FMLP and PMA was enhanced by ~ 150 and 50%, respectively. Botulinum C2 toxin blocked both random migration of neutrophils and migration induced by FMLP, complement C5a, leukotriene B_4, and a novel monocyte-derived chemotactic agent. The data suggest that botulinum C2 toxin-catalyzed ADP-ribosylation of a minor actin pool has a pronounced effect on the activation of human neutrophils by various stimulants.

Introduction

Activation of neutrophils by chemotactic agents induces cellular responses like cell shape change, migration, degranulation, and phagocytosis (1). All these events depend on cellular motile functions and the dynamic processes involved in restructuring of the cytoskeleton (1, 2). Recent studies have indicated that actin, besides its role in muscle contraction, is basically involved in cellular motile processes of such nonmuscle cells as neutrophils and platelets (2, 3). In nonmuscle cells, these motile functions are apparently associated with changes in the state of actin polymerization. It thus has been shown that activation of neutrophils or platelets is accompanied by a rapid increase in polymerized actin (3–5). Moreover, agents that block actin polymerization, like cytochalasins (6), largely interfere with leukocyte activation caused by various chemotactic stimulants (7–9). Not only phenomena obviously related to cell motile functions are regulated by cytoskeletal elements. Several findings indicate an involvement of cytoskeleton proteins in activation of NADPH oxidase by chemotactic agents (7–10). Again, this conclusion was mainly drawn from the findings that cytochalasins drastically increased the O_2^- production caused by neutrophil stimulatory agents like FMLP, platelet-activating factor (PAF),[1] and Con A (7–9).

Botulinum C2 toxin belongs to a class of bacterial ADP-ribosyltransferases that modify actin (11, 12). The toxin is binary in structure and consists of components I (C2 I) and II (C2 II) (13). Whereas C2 I (M_r 50,000) possesses ADP-ribosyltransferase activity, C2 II (M_r 100,000) is apparently involved in the binding of the toxin to the cell membrane. The substrate of botulinum C2 toxin is nonmuscle G actin, but not polymerized F actin (12, 14). Moreover, the covalent modification of actin blocks the ability of actin to polymerize (11, 12, 15). This ability to inhibit actin polymerization thus qualifies botulinum C2 toxin as a new tool with which to study the role of actin in various cell functions. Therefore, these results prompted us to study the effects of botulinum C2 toxin on activation of human neutrophils by various stimulants and compare its effects with those of cytochalasin B.

Methods

Materials. FMLP, SOD, cytochalasin B, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), phorbol 12-myristate 13-acetate (PMA), thymidine, leupeptin, *p*-nitro-phenyl-*N*-acetyl-D-glucosamide, and PMSF were obtained from Sigma Chemical (Deisenhofen, FRG). Dextran T 500 and Ficoll-Hypaque were purchased from Pharmacia Fine Chemicals (Freiburg, FRG); ATP from Boehringer Mannheim-GmbH (Mannheim, FRG); and Triton X-100 from SERVA Feinbiochemical & Co. (Heidelberg, FRG). Xylol and toluene were obtained from Roth GmbH & Co. KG (Karlsruhe, FRG); [^{57}Co]-cyanocobalamine and [^{32}P]NAD from Amersham Buchler (Braunschweig, FRG); and Con A from Miles Laboratories, Inc. (Munich, FRG). Leukotriene B_4 (LTB_4), the chemotactic split peptide C5a, and the monocyte-derived chemotactic agent (MOC) were produced as described (16). Botulinum C2 toxin was prepared and activated essentially as described (13). Pertussis toxin was kindly donated by Dr. Yajima (Kobe, Japan). 3-μm-pore size cellulose nitrate filters were purchased from Sartorius GmbH (Göttingen, FRG).

Preparation of human neutrophils. To determine O_2^- production and for the ADP-ribosylation assay, human neutrophils were isolated from freshly drawn venous blood obtained from healthy donors, essentially as described by Markert et al. (17). To determine migration

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Received for publication 9 February 1988 and in revised form 20 May 1988.

J. Clin. Invest.
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0021-9738/88/10/1376/07 $2.00
Volume 82, October 1988, 1376–1382

1. *Abbreviations used in this paper:* C2 I, component I of botulinum C2 toxin; C2 II, component II of botulinum C2 toxin; MOC, monocyte-derived chemotactic agent; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine).

and enzyme release, neutrophils were isolated as described (16). Either isolation procedure resulted in 95% of neutrophils with viability > 95%, as measured by trypan blue exclusion. Toxin treatment of neutrophils for up to at least 150 min did not reduce viability of cells.

ADP-ribosylation assay. ADP-ribosylation was performed essentially as described (12, 15). Briefly, neutrophils (10^7 cells/ml) were incubated with 400 ng/ml C2 I and 1.6 μg/ml C2 II of botulinum toxin in a buffer containing 138 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes, pH 7.5, for the indicated periods of time. Thereafter, the cells were washed twice; lysed in a medium containing 10 mM triethanolamine-HCl (pH 7.5), 0.5 mM PMSF, 0.1 μg/ml leupeptin, and 25 mM EDTA; frozen; thawed; homogenized; and finally used in the ADP-ribosylation assay. ADP-ribosylation of neutrophil lysates (~ 1 mg of protein/tube) was carried out in a buffer containing 50 mM triethanolamine-HCl (pH 7.4), 10 mM thymidine, 0.5 mM ATP, 4 mM $MgCl_2$, 1 μM [^{32}P]NAD (~ 1 μCi), 0.02% BSA (wt/vol) and 1 μg/ml botulinum C2 toxin component I. The incubation was carried out for 10 min at 37°C. The reaction was stopped by the addition of 20 μl of a buffer containing 10% (wt/vol) SDS, 10% (wt/vol) saccharose, 1% (vol/vol) 2-mercaptoethanol, and 100 mM Tris-HCl, pH 7.4. Thereafter, the radioactively labeled proteins were analyzed by SDS-PAGE according to Laemmli (18). Quantitative determination of the incorporated [^{32}P]ADP-ribose was performed by terminating the ADP-ribosylation reaction with 400 μl of a solution containing 2% SDS (wt/vol) and 1 mg/ml BSA and precipitating the proteins with 500 μl of TCA (30%, wt/vol). Thereafter, the precipitated proteins were collected on nitrocellulose filters. The filters were washed 10 times with 2 ml of 6% TCA, placed in scintillation fluid, and counted for retained radioactivity.

Superoxide anion (O_2^-) production. Superoxide anion (O_2^-) production was measured as SOD-inhibitable reduction of ferricytochrome *C* at 550 nm according to Markert et al. (17) in the same buffer as described for toxin treatment of neutrophils. For discontinuous determination of O_2^- production, the reaction was started with the addition of the indicated concentrations of various stimulants. After 15 min of incubation at 37°C, the reaction tubes were centrifuged and the superoxide-dependent cytochrome C reduction was measured. Continuous measurement of O_2^- formation was performed with a photometer and plotted by means of enzyme-kinetic software (LKB Instruments, Gaithersburg, MD).

Release of N-acetyl-glucosaminidase and vitamin B12-binding protein. The assays were performed in HBSS (pH 7.2) with 0.5% BSA. The reaction was started by the addition of 0.1 μM FMLP or 5 ng/ml PMA and continued for 5 min at 37°C. Thereafter, the cells (10^6) were centrifuged for 5 min at 250 *g* and the supernatants were analyzed for *N*-acetyl-glucosaminidase activity and for vitamin B12-binding protein release as described by Kownatzki et al. (19) and Fehr et al. (20), respectively. For calculation of total cellular *N*-acetyl-glucosaminidase and vitamin B12-binding protein content, neutrophils were lysed with 0.1% Triton X-100 and treated as described above.

Cell migration. Migration of neutrophils (10^6) was measured with the double-chamber filter method using blind well chambers (Neuro Probe, Inc., Cabin John, MD) as described (16).

Protein concentrations were determined according to Lowry et al. (21).

Results

Influence of botulinum C2 toxin on O_2^- production. At first we compared the effects of cytochalasin B and botulinum C2 toxin on O_2^- production of human neutrophils. Fig. 1 *A* shows the well-known phenomenon that cytochalasin B increases the stimulation of O_2^- production by FMLP. In the absence of cytochalasin B, 0.1 μM FMLP caused the formation of ~ 10 nmol of $O_2^-/10^6$ cells. Preincubation of neutrophils with 2 μg/ml cytochalasin B for 2 min increased the FMLP-stimulated O_2^- production by ~ 100%, apparently without changing the half-maximally effective concentration of FMLP. Pretreatment of neutrophils for 45 min with botulinum C2 toxin (400 μg C2 I and 1.6 μg C2 II) caused an almost identical increase in O_2^- production without major alteration of the FMLP concentration-response curve (Fig. 1 *B*). As found with cytochalasin B, botulinum C2 toxin did not influence O_2^- production in the absence of FMLP. Under these conditions, the effects of both toxins were not additive, as pretreatment of neutrophils with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 45 min and additional preincubation with cytochalasin B did not further increase the FMLP-induced O_2^- production (not shown). As shown in Table I, the stimulatory effect of botulinum C2 toxin depended on the presence of both components of the toxin. C2 II slightly increased O_2^- production, which was probably due to a slight contamination of the

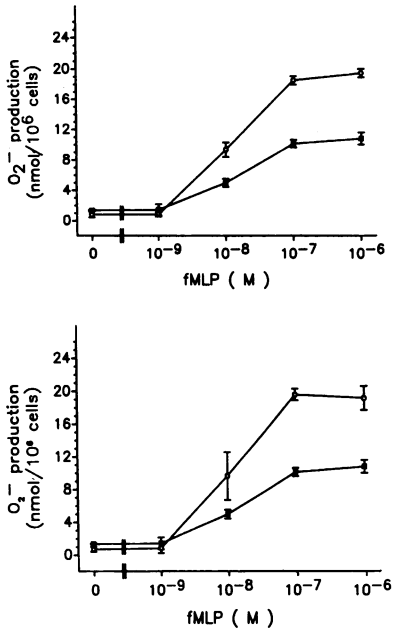

Figure 1. FMLP concentration dependence of O_2^- production by human neutrophils. (*Top*) Influence of cytochalasin B. Human neutrophils (10^6 cells) were preincubated with (○) and without (×) 2 μg/ml cytochalasin B for 2 min. Treated neutrophils were stimulated with the indicated concentrations of FMLP for 15 min and thereafter O_2^- production was determined as described. (*Bottom*) Influence of botulinum C2 toxin. Human neutrophils (10^7 cells) were preincubated with (○) and without (×) botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 45 min. Aliquots (10^6 cells) of treated neutrophils were stimulated with the indicated concentrations of FMLP for 15 min and O_2^- production was determined. Data given are means±SEM of four separate experiments performed in triplicate.

Table I. Influences of Botulinum C2 Toxin Components on FMLP-stimulated O_2^- Production by Human Neutrophils

Toxin	FMLP-stimulated O_2^- production
	nmol/10^6 cells
Control	8.6 (±2.4)
C2 I	8.5 (±2.1)
C2 II	11.7 (±3.8)
C2 I + C2 II	22.0 (±2.2)

Human neutrophils (10^7 cells) were treated without and with botulinum C2 toxin I (400 ng/ml), C2 II (1.6 μg/ml), or C2 I plus C2 II for 45 min. Aliquots (10^6 cells) of treated neutrophils were stimulated with 0.1 μM FMLP for 15 min and O_2^- production was determined as described. Data given are means ± SEM of three separate experiments performed in triplicate.

binding component of botulinum C2 toxin with C2 I, which by itself had no effect. Fig. 2 shows the effects of botulinum C2 toxin treatment on the FMLP-stimulated O_2^- production as determined by continuous monitoring. Toxin treatment increased the rate and the duration of O_2^- production, leading to an approximately twofold increase overall in O_2^- accumulation. Fig. 3 illustrates the time dependence of botulinum C2 toxin's action. The effect of the toxin was observed after a latency period, whose duration depended on the concentration of the toxin. While in the presence of 400 ng/ml C2 I and 1.6 μg/ml C2 II, the maximum toxin effect occurred after 45 min of incubation, at a fourfold lower concentration of both components, maximum increase in FMLP-stimulated O_2^- production was observed only after ~ 90 min of incubation. To study whether the effects of botulinum C2 toxin and cytochalasin B interfere with each other in greater detail, we treated neutrophils with submaximal concentrations of either toxin for various periods of time. As shown in Fig. 4, pretreatment of neutrophils with 0.015 and 2 μg/ml cytochalasin B increased FMLP-stimulated O_2^- production ~ 3.3 and 5.5-fold, respectively. Pretreatment of the cells with botulinum C2 toxin (200 ng/ml C2 I and 800 ng/ml C2 II) for 10 min enhanced stimulated O_2^- production about twofold. Further addition of cytochalasin B (0.015 μg/ml for 2 min) caused an approximate fivefold increase in O_2^- formation. Enhancement of O_2^- production by botulinum C2 toxin and cytochalasin B, although not additive at maximum effective concentrations of either toxin, thus were additive at submaximal concentrations. Botulinum C2 toxin apparently elicits its effects on eukaryotic cells by an ADP-ribosylation of actin (12). Therefore, we studied whether actin was also ADP-ribosylated by botulinum C2 toxin in intact neutrophils. For this purpose, neutrophils were treated with botulinum C2 toxin for up to 3 h. At the indicated periods of time, toxin-treated neutrophils were lysed and a second ADP ribosylation was performed in the presence of [^{32}P]NAD. The autoradiogram in Fig. 5 *A* illustrates that in neutrophil lysates, a 43-kD protein was labeled by botulinum

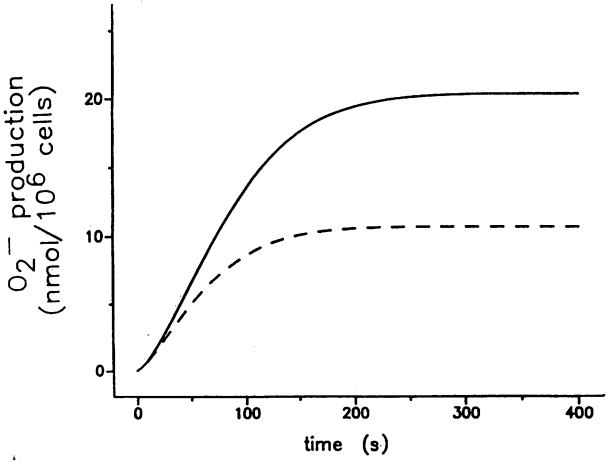

Figure 2. Continuous monitoring of FMLP-stimulated O_2^- production of human neutrophils. Neutrophils (10^7 cells) were treated with (——) and without (– – –) botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 45 min. Thereafter aliquots (10^6 cells) of treated neutrophils were stimulated by the addition of 0.1 μM FMLP and O_2^- production was continuously determined. The data given are from a typical experiment repeated three times with identical results.

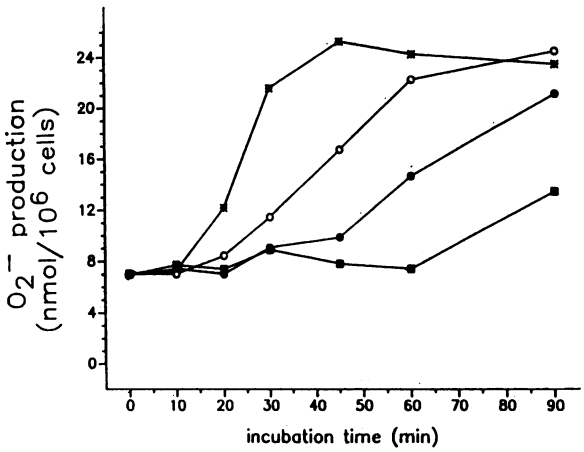

Figure 3. Botulinum C2 toxin concentration and time dependence of FMLP-stimulated O_2^- production by neutrophils. Human neutrophils (10^7 cells) were treated with botulinum C2 toxin: 400 and 1,600 ng/ml (×), 200 and 800 ng/ml (○), 100 and 400 ng/ml (●), and 40 ng/ml C2I and 160 ng/ml C2II (■), respectively, for the indicated periods of time. Aliquots (10^6 cells) of treated neutrophils were stimulated with 0.1 μM FMLP for 15 min and O_2^- production was determined. Data given are means of triplicate determinations (SD < ±1.8 nmol $O_2^-/10^6$ cells) of a typical experiment repeated four times with identical results.

C2 toxin. In lysates of neutrophils pretreated with botulinum C2 toxin, the labeling was largely decreased, suggesting that actin had been modified during the pretreatment period. However, whereas FMLP-induced O_2^- production was maximally enhanced after 45 min of toxin treatment, only a small fraction (~ 20%) of the cellular actin pool was ADP-ribosylated in intact neutrophils (Fig. 5 *B*).

Next we studied the effects of botulinum C2 toxin on stimulation of O_2^- production by PAF, Con A, and PMA. As shown in Table II, 0.1 μM FMLP, 5 μM PAF, 50 μg/ml Con A, and 30 ng/ml PMA caused the production of ~ 8, 4, 4, and 22 nmol $O_2^-/10^6$ cells, respectively. Pretreatment of neutrophils with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 45 min increased the stimulatory effects of FMLP, PAF, and Con A by ~ 170, 160, and 280%, respectively. In contrast, stimulation of O_2^- production by PMA was not affected by the

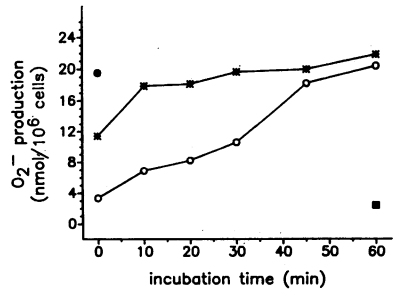

Figure 4. Influence of submaximal concentrations of cytochalasin B and botulinum C2 toxin on FMLP-stimulated O_2^- production. Neutrophils (10^7 cells) were treated without (● and ■) and with (○ and ×) botulinum C2 toxin (200 ng/ml C2 I and 800 ng/ml C2 II) for the indicated periods of time. Aliquots (10^6 cells) of treated neutrophils were incubated with 0.015 μg/ml (×) or 2 μg/ml (●) of cytochalasin B for 2 min. Thereafter, cells were immediately stimulated with 0.1 μM FMLP for 15 min and O_2^- production was determined as described. The data given are means of triplicate determination (SD < ±1.75 nmol $O_2^-/10^6$ cells) of a typical experiment repeated twice with identical results.

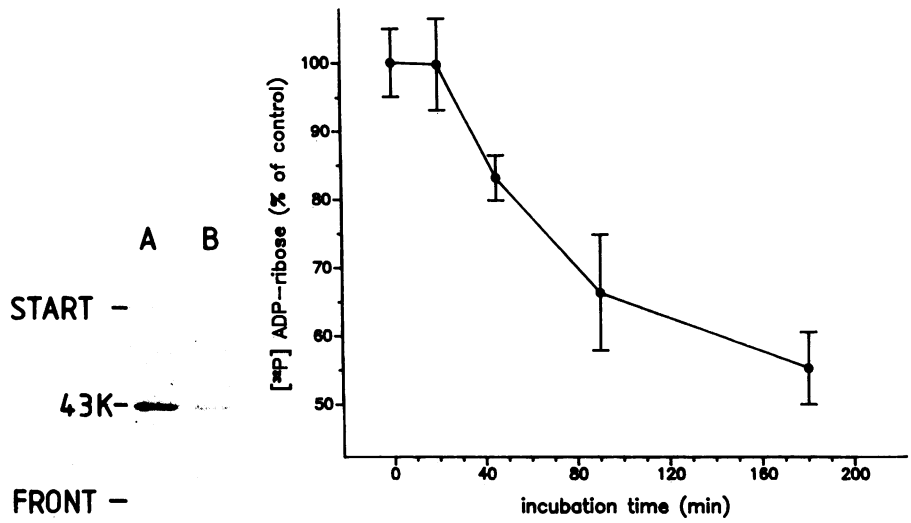

Figure 5. ADP-ribosylation of human neutrophil proteins by botulinum C2 toxin. (*A*) Autoradiography of [^{32}P]ADP-ribosylated neutrophil proteins. Human neutrophils (10^7 cells/ml) were treated without (lane *A*) and with (lane *B*) botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 180 min. Thereafter, the cells were washed and lysed and the lysate was ADP-ribosylated with 1 μg/ml botulinum C2 toxin C2 I in the presence of 0.5 μM [^{32}P]NAD as described. Radioactively labeled proteins were analyzed by SDS-PAGE. The autoradiogram of the gel is shown. (*B*) Time course of ADP-ribosylation of neutrophil proteins by botulinum C2 toxin. Human neutrophils (10^7 cells/ml) were treated with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for the indicated periods of time. Thereafter the cells were washed and lysed and [^{32}P]ADP-ribosylation of the lysate was performed as described above. Quantitative measurements of radioactively labeled proteins were performed by the filtration assay. The data given are means (±SD) of triplicate determination of a typical experiment repeated three times with identical results.

toxin. This was also observed when submaximal concentrations of PMA (0.5 ng/ml) were used (not shown).

Recently, it has been suggested that cytochalasin B may act on stimulated O_2^- production by interfering with desensitization processes, which rapidly reduce responsiveness towards FMLP (7). Therefore we studied whether botulinum C2 toxin may also affect desensitization processes elicited by FMLP receptor occupation. For this purpose, neutrophils were pretreated with increasing concentrations of FMLP for 7.5 min, i.e., until O_2^- production had ceased (see continuous determination of O_2^- formation, Fig. 2). Thereafter, 0.1 μM FMLP was readded and the O_2^- production was determined. Fig. 6 shows that prior treatment of neutrophils with FMLP reduced subsequent stimulation of O_2^- production in a concentration-dependent manner. Pretreatment of neutrophils with botulinum C2 toxin largely prevented FMLP-dependent reduction in O_2^- production.

Influences of botulinum C2 toxin on secretion of N-acetyl-glucosaminidase and vitamin B12-binding protein. The effects of botulinum C2 toxin on *N*-acetyl-glucosaminidase secretion after various periods of incubation time are shown in Fig. 7. Even incubation of neutrophils with botulinum C2 toxin for up to 90 min did not cause any secretion of *N*-acetyl-glucosaminidase in the absence of stimulants. In contrast, FMLP-induced release of this enzyme clearly increased after 45 min of incubation in the presence of the toxin. The release of *N*-acetyl-glucosaminidase caused by 5 ng/ml PMA was much less increased by toxin treatment. As found for *N*-acetyl-glucosaminidase secretion, FMLP-stimulated release of vitamin B12-binding protein, a component of specific granules, was largely increased (~ 150%) by botulinum C2 toxin (Fig. 8). The effect of PMA, which released vitamin B12-binding protein much more effectively than *N*-acetyl-glucosaminidase, was increased by ~ 50% with botulinum C2 toxin. As found for O_2^- production both components (C2 I and C2 II) were required to see these toxin's actions (not shown).

Influence of botulinum C2 toxin on migration of neutrophils. The influence of botulinum C2 toxin on the migration of neutrophils stimulated with FMLP, C5a, LTB_4, and a monocyte-derived chemotactic agent (MOC) is shown in Table III.

Table II. Effects of Botulinum C2 Toxin on O_2^- Production by Human Neutrophils Stimulated with FMLP, PAF, Con A, and PMA

Stimulants	O_2^- production	
	Control	C2 toxin
	nmol/10^6 cells	
FMLP	7.7 (±3.5)	20.8 (±0.6)
PAF	3.6 (±1.3)	9.2 (±0.9)
Con A	3.8 (±1.3)	14.5 (±3.0)
PMA	21.1 (±1.3)	20.4 (±0.4)

Human neutrophils (10^7 cells) were treated with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 45 min. Aliquots (10^6 cells) of treated neutrophils were stimulated with 0.1 μM FMLP, 5 μM PAF, 50 ng/ml Con A, and 30 ng/ml PMA for 25 min and O_2^- production was determined as described. Data given are means ± SEM of three separate experiments performed in triplicate.

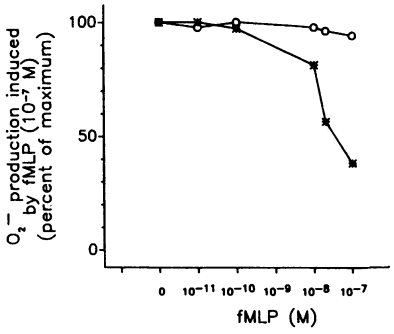

Figure 6. Desensitization of FMLP-stimulated O_2^- production by human neutrophils. Neutrophils were preincubated with (○) or without (*) botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 I) for 60 min. Aliquots (10^6 cells) of treated neutrophils were stimulated with the indicated concentrations of FMLP for 7.5 min. Thereafter, neutrophils were again stimulated with 0.1 μM FMLP and O_2^- production was determined as described. Maximal O_2^- production, achieved in the absence of FMLP during the prestimulation period, was 8.2 and 17.9 nmol $O_2^-/10^6$ cells without and with botulinum C2 toxin, respectively, and is indicated as 100%. Data given are means of triplicate determinations of a typical experiment repeated four times with identical results.

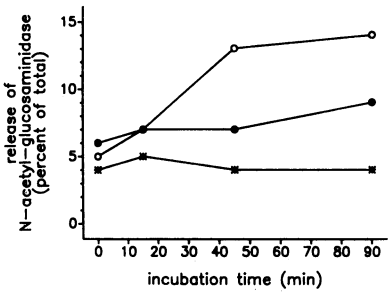

Figure 7. Influences of botulinum C2 toxin on FMLP- and PMA-stimulated *N*-acetyl-glucosaminidase release from human neutrophils. Human neutrophils (10^7 cells) were treated with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for the indicated periods of time. Thereafter, aliquots (10^6 cells) of treated neutrophils were stimulated without (*) and with 0.1 μM FMLP (○) or 5 ng/ml PMA (●) for 5 min and the release of *N*-acetyl-glucosaminidase was determined as described. The data given are means of triplicate determinations of a typical experiment repeated four times with identical results.

All these chemotactic agents increased migration of leukocytes almost to the same extent at the concentrations used. After treatment of neutrophils for 60 min with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II), migration was completely inhibited whether chemotactic agents were present or not.

Discussion

The binary botulinum C2 toxin is known to ADP-ribosylate actin, a modification that blocks the ability of actin to polymerize (11, 12, 15). Here we show that botulinum C2 toxin effectively interferes with activation of human neutrophils. The toxin largely increased the O_2^- production stimulated by various chemotactic agents such as FMLP, PAF, and Con A. The toxin effects on O_2^- production and secretion occurred with some latency, depended on the presence of both components of botulinum C2 toxin, and were observed only in the presence of neutrophil stimulants but not in their absence, indicating the specificity of the toxin action. These data are in agreement with the effects of the toxin on O_2^- consumption and luminol-dependent chemoluminescence in rat neutrophils reported recently (22). Furthermore, as shown with other cell types (12, 15), botulinum C2 toxin was able to ADP-ribosylate actin in intact neutrophils. It thus is feasible that the ADP-ribosylation of actin by botulinum C2 toxin is the molecular basis for the altered neutrophil response. This hypothesis is corroborated by the observation that cytochalasin B, another agent that blocks actin polymerization (6), alters the response towards various chemotactic agents (7–9). Moreover, the findings reported herein, that both botulinum C2 toxin and cytochalasin B were additive at submaximally but not at maximally effective concentrations, support the view that both toxins act via similar pathways. Although there exists a growing amount of evidence that actin polymerization is somehow involved in stimulation of O_2^- production, the precise contribution of the cytoskeleton protein to this action is still unknown. Various molecular levels are conceivable as sites for an involvement of actin in signal response coupling of O_2^- production. It is generally accepted that stimulation of NADPH oxidase by FMLP involves a pertussis toxin-sensitive G protein (23). We have observed that ADP-ribosylation of actin by botulinum C2 toxin does not affect the blockade of the FMLP-stimulated O_2^- production by pertussis toxin (not shown) indicating that the involved G protein is not bypassed by ADP-ribosylation of actin. The observation that botulinum C2 toxin did not enhance stimulation of O_2^- production by PMA suggests that toxin action does not enhance stimulation of NADPH oxidase by activated protein kinase C (10).

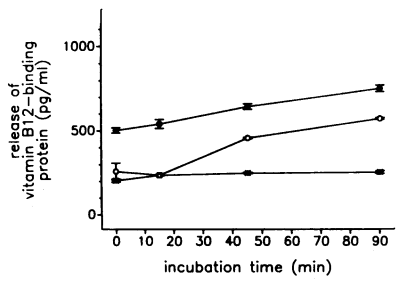

Figure 8. Influences of botulinum C2 toxin on FMLP- and PMA-stimulated vitamin B12 binding-protein release from human neutrophils. Human neutrophils (10^7 cells) were treated with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for the indicated periods of time. Thereafter aliquots (10^6 cells) of treated neutrophils were stimulated without (*) and with 0.1 μM FMLP (○) or 5 ng/ml PMA (●) for 5 min and the release of vitamin B12-binding protein was determined as described. The data given are means±SD of triplicate determinations of a typical experiment repeated twice with identical results.

Table III. Effect of Botulinum C2 Toxin on the Chemotactic Response of Human Neutrophils to Various Chemoattractants

Stimulants	Migration	
	Control	C2 toxin
	μm/90 min	
None	58 (±3)	0
FMLP	130 (±4)	0
C5a	117 (±10)	0
LTB_4	120 (±8)	0
MOC	128 (±8)	0

Human neutrophils (10^7 cells) were treated without and with botulinum C2 toxin (400 ng/ml C2 I and 1.6 μg/ml C2 II) for 60 min. Migration of treated neutrophils (10^6 cells) was determined after 90 min with no stimulation (none) and stimulated with 5 nM FMLP, C5a, LTB_4, and MOC. All three used at the lowest concentration yielding optimum migration.

It has been shown that cytoskeletal elements play key roles in the dynamics of FMLP receptors. Jesaitis et al. (7, 24) demonstrated that the ligand-occupied FMLP receptor associates very rapidly with the cytoskeleton. This process is supposedly involved in desensitization of FMLP-stimulated O_2^- production (7) and is reportedly blocked by cytochalasins (7, 24). Here we demonstrate that botulinum C2 toxin effectively reduced desensitization of FMLP-stimulated O_2^- production achieved by pretreatment of neutrophils with increasing concentrations of FMLP, a finding that supports the hypothesis that polymerization of actin is involved in receptor desensitization (7).

The continuous monitoring of FMLP-stimulated O_2^- production reported herein showed an increase in duration and velocity of NADPH oxidase after botulinum C2 toxin treatment. These data could be explained by a decrease in receptor disappearance rate. Another explanation for toxin-induced enhancement of O_2^- production could be that botulinum C2

toxin increased the reappearance of active membrane receptors out of certain compartments of the cell into the membrane. This recycling may be associated with fusion of vesicles with the plasma membrane, a process supposedly involved in enhancement of O_2^- production by cytochalasin B (25).

Interestingly, maximum toxin effects on O_2^- production were observed even when only a minor fraction of the cellular actin pool was ADP-ribosylated by botulinum C2 toxin. At least two explanations are conceivable: first, that a small, functionally specified pool of actin has to be modified to elicit augmentation of O_2^- production. This pool, supposedly located near the membrane and involved in receptor dynamics, may be easily accessible for the toxin. Second, there exists evidence that actin ADP-ribosylated by *Clostridium perfringens* iota toxin, which modifies actin at the same site as botulinum C2 toxin (26), behaves like a capping protein (27). If ADP-ribosylated neutrophil actin has capping protein function, a small amount of modified actin would be sufficient to block rapid polymerization occurring during activation of neutrophils.

Botulinum C2 toxin effectively increased the release of *N*-acetyl-glucosaminidase and vitamin B12-binding protein stimulated by FMLP, whereas basal release of neither granule component was affected by the toxin. These observations can be interpreted to indicate that actin is involved in release reactions of both azurophilic and specific granules of neutrophils and agree with reports showing that cytochalasin B increased the release of these vesicle components in neutrophils (28, 29). Interestingly, stimulation of secretion by PMA was also enhanced by botulinum C2 toxin. Botulinum C2 toxin thus affected PMA-induced stimulation of O_2^- production and stimulation of secretion differently. This finding might indicate that separate mechanisms of actin are involved in regulation of both processes in neutrophils. On the other hand, it is feasible that degranulation enhanced by ADP-ribosylation permits expression of membrane receptors, thereby enhancing FMLP-induced, but not PMA-induced O_2^- production. It has been suggested that actin is involved in secretory and exocytotic processes of various cell types like chromaffin cells (30) and mast cells (31). Recent studies on the influence of botulinum C2 toxin on secretion of rat mast cells have shown that stimulation of histamine release by compound 48/80 or PMA was not enhanced, but rather inhibited by toxin treatment (31). Although treatment of mast cells with botulinum C2 toxin differed somewhat from the toxin treatment reported herein, these data suggest that actin involvement in secretion is largely different in various cell types.

In contrast to O_2^- production and granule release, migration of neutrophils was completely inhibited by botulinum C2 toxin treatment. Even random migration of neutrophils was completely suppressed. Again, these results confirm the results of recent studies with cytochalasin (2) and support the notion that actin is involved in migration of neutrophils. Whereas actin evidently plays a modulatory role in O_2^- production and secretion, migration of neutrophils thus apparently completely depends on the unimpaired ability of actin to polymerize.

Botulinum C2 toxin increased O_2^- production and secretion but inhibited migration of neutrophils stimulated by various chemotactic agents or neutrophil stimulants. These toxin effects are probably due to ADP-ribosylation of actin, which renders the protein unable to polymerize and to form microfilaments. Furthermore, the data presented suggest that polymerization and depolymerization of specific actin pools are basically involved in the cascade of events occurring alongside neutrophil activation. Besides cytochalasins, whose use is hampered by questionable specificity (6), botulinum C2 toxin thus appears to be a novel, specific tool to study the role of actin in signal-response coupling of neutrophils and various other cell types.

Acknowledgments

We thank Dr. K. H. Jakobs and Dr. McLeish for very helpful discussions and Dr. G. Koch for assistance in preparation of drawings. The excellent technical assistance of Mrs. M. Laux and Mrs. S. Uhrich is gratefully acknowledged.

This work was supported by the Deutsche Forschungsgemeinschaft (Ak 6/1-2). Material described herein is an essential part of the thesis of J. Norgauer.

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